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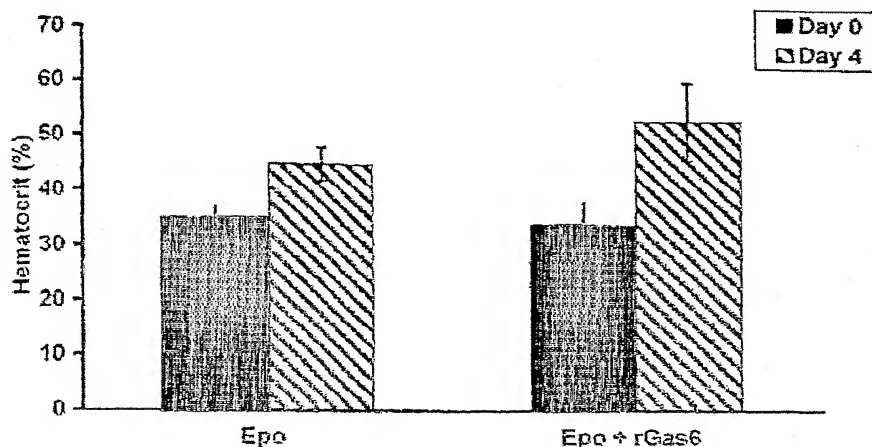
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[Continued on next page]

(54) Title: TREATMENT OF ANEMIA



Epo: 10 IU/day

rGas6: 2 microgram/day

(57) Abstract: The present invention relates to a method for the treatment of anemia based on the administration of the product of growth arrest-specific gene 6 (Gas6), its mutants, variants, active derivatives and the physiological tolerated salts of said Gas6 derivatives or of a group of biologically active substances inducing a GAS6 expression or releasing action or of a group of compounds that activate the Sky, Axl or Mer receptor tyrosine kinases. Furthermore the invention relates to a pharmaceutically effective composition to treat anemia. The composition comprises a pharmaceutically effective amount of a product of growth arrest-specific gene 6 (Gas6), or its mutant, variant, active derivative or the physiological tolerated salts of said Gas6 derivative or a biologically active substance that induces GAS6 expression or GAS6 release or a substance that activates the Sky, Axl or Mer receptor tyrosine kinases.



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TREATMENT OF ANEMIA

Field of the invention

The present invention relates to formulations and methods for the treatment or prevention or cure of anemia in humans or mammals and for the manufacture of a medicament to treat a human or mammalian patient to prevent, reduce or cure anemia of said patient. In particular, the anemia can be induced by hemolysis or caused by or associated with various disorders such as chronic disease, chronic renal failure, aplastic anemia, hemolytic anemia, malignancies, endocrine deficiencies or can be caused by a specific treatment such as chemotherapy.

Background of the invention

The term *anemia* refers to a reduction below normal in hemoglobin level or red blood cells in the blood. Anemia is a major cause of morbidity and mortality. It may result from a reduced rate of production or increased rate of destruction of red blood cells or their loss from the circulation by bleeding or from defective red blood cells as in sickle cell anemia. Although the cause of anemia can be a primary disorder of the production or survival of red blood cells, in many cases it is secondary to diseases of other systems (Weatherall & Provan (2000) *Lancet* **355**, 1169-1175).

The mature red blood cell has two main functions. First, it must survive in the circulation for as long as possible, most of the time in vessels smaller than its own diameter. Second, it must both preserve its hemoglobin in state suitable for oxygen transport and adapt the amount of oxygen that is delivered to the needs of the tissues (Weatherall & Provan, cited above).

The process by which erythroid cells are produced is called erythropoiesis. The production of mature red blood cells from pluripotent hematopoietic stem cells requires the coordinated action of different cytokine signaling pathways to assure controlled cell proliferation, survival, differentiation and death. Under normal conditions, the whole process of erythropoiesis results in a red blood production rate such that the red cell mass in the body is kept

constant. Erythropoiesis involves a great variety and number of cells at different stages of maturation, starting with the first stem cell progeny committed to erythroid differentiation and ending with the mature circulating red blood cell. Erythropoiesis occurs in distinct stages and anatomic sites during development.

5 Primitive erythropoiesis begins around embryonic day 7 when red blood cells originate in the blood islands of the yolk sac. These cells express embryonic globins and are not dependent upon erythropoietin (EPO) – [Wu *et al.* (1995) *Cell* **83**, 59-67; Lin *et al.* (1996) *Genes Dev.* **10**, 154-164]. EPO is the major cytokine for definitive and adult erythropoiesis. Definitive erythropoiesis starts at
10 approximately day 10 when the site of red blood cell production shifts from the yolk sac to the foetal liver and is characterised by the production of non-nucleated erythrocytes expressing adult globin genes. At birth, erythroblastic islands in the bone marrow and the red pulp of the spleen become dominant sites of red blood cell production.

15 Epo also regulates the compensatory erythropoietic response to reduced tissue oxygen tension (hypoxia) and anemia. In humans, elevated Epo levels promote recovery from anemia by stimulating the generation of erythroid progenitors in the bone marrow and spleen. In the mouse, Epo enhances erythropoiesis primarily in the spleen.

20 Recombinant Epo is in widespread clinical use to treat anemia associated with a variety of disorders that include, for example, chronic renal failure, myelodysplastic syndrome, rheumatoid arthritis, and bone marrow transplantation, often resulting in substantial improvements in quality of life and prolonged survival (Winearls *et al.* (1986) *Lancet* **2(8517)**, 1175-1178.;
25 Eschbach *et al.* (1987) *N Engl J Med* **316**, 73-78). However, Epo is not uniformly effective, with many individuals being entirely refractory to even high doses (Horl *et al.* (2000) *Nephrol Dial Transplant* **15**, 43-50). Over 50% of patients with cancer have an inadequate response to Epo, approximately 10% with end-stage renal disease are hyporesponsive (Glaspy *et al.* (1997) *J Clin Oncol* **15**, 1218-1234; Demetri *et al.* (1998) *J Clin Oncol* **16**, 3412-3425), and
30 less than 10% with myelodysplastic syndrome respond favourably (Estey (2003) *Curr Opin Hematol* **10**, 60-67). Although several factors, such as inflammation

(Horl *et al.* (2000) cited above), iron and vitamin deficiency, inadequate dialysis, aluminium toxicity, and hyperparathyroidism (Macdougall (1995) *Nephrol Dial Transplant* **10**, 607-614; Muirhead *et al.* (1995) *Am J Kidney Dis* **26**, S1-24; Nitta *et al.* (2002) *Acta Haematol* **108**, 168-170; Goicoechea *et al.* (1998) *Kidney Int* **54**, 1337-1343) have been identified that predict for a poor therapeutic response, the molecular mechanisms of resistance to Epo remain largely obscure.

Moreover, adverse effects and contraindications for treatment with Epo have been determined. Over-treatment with the hormone in patients undergoing hemodialysis has led to excessive increases in the hematocrit, leading to the aggravation of hypertension (sometimes leading to hypertensive encephalopathy and headaches) or thrombotic complications (Horl *et al.* (2000) *Nephrol Dial Transplant* **15**, 51-55).

Gas6, the product of the growth arrest-specific gene 6 (*gas6*), is a new member of the vitamin K-dependent protein family (Manfioletti *et al.* (1993) *Mol Cell Biol.* **13**, 4976-4985; Schneider *et al.* (1988) *Cell* **54**, 787-793). Proteins belonging to this family are characterised by post-translational γ -carboxylation of certain glutamic acid residues by a carboxylase, using vitamin K as cofactor. The γ -carboxyglutamic acid (Gla)-containing module in prothrombin, coagulation factors VII, IX and X, protein C, protein S, protein Z and Gas6 allow these vitamin K-dependent proteins to bind to negatively charged phospholipid membranes (Dahlback (2000) *Lancet* **355**, 1627-1632). Gas6 is structurally similar to protein S, but lacks a loop, crucial for the anticoagulant activity of protein S (Manfioletti *et al.* cited above). The latter is a cofactor for activated protein C, which inactivates the coagulation factors Va and VIIIa (Dahlback (1991) *Thromb Haemost* **66**, 49-61). Genetic deficiency of protein S in man is one of the most severe inherited risk factors for thrombosis (Borgel *et al.* (1997) *Thromb Haemost.* **78**, 351-356).

Apart from a Gla-domain-dependent interaction with phospholipid membranes (Nakano *et al.* (1997) *J Biol Chem.* **272**, 29411-29414). Gas6 also binds as a ligand to the receptor tyrosine kinases Axl (Ark, Ufo, Tyro7), Sky (Rse, Tyro3, Dtk, Etk, Brt, Tif) and Mer (c-Mer, Eyk, Nyk) (Varnum *et al.* (1995)

Nature **373**, 623-626; Godowski *et al.* (1995) *Cell* **82**, 355-358; Nagata *et al.* (1996) *J Biol Chem.* **271**, 30022-30027; Crosier *et al.* 1997, *Pathology* **29**, 131-135; Chen J *et al.* 1997, *Oncogene* **14**, 2033-2039) by its carboxy-terminal globular G domains (Nagata (1996) cited above). It has been implicated in reversible cell growth arrest (Manfiotti *et al.* (1993) cited above; Schneider *et al.* 1988, cited above), survival (Goruppi *et al.* 1996, *Oncogene* **12**, 471-80), proliferation (Goruppi *et al.* cited above, Li *et al.* (1996) *J Neurosci.* **16**, 2012-2019; Nakano *et al.* (1995) *J Biol Chem.* **270**, 5702-5705), and cell adhesion (Nakano *et al.* (1997) cited above; Fridell *et al.* (1998) *J Biol Chem* **273**, 7123-7126, McCloskey *et al.* (1997) *J Biol Chem* **272**, 23285-23291). Mice with a triple deficiency of Axl, Sky and Mer are viable, but have not been reported to suffer spontaneous bleeding or thrombosis (Lu *et al.* (1999) *Nature* **398**, 723-8).

It has been shown that Gas6 deficient (Gas6^{-/-}) mice, generated by homologous recombination, are born at the expected mendelian frequency. Gas6^{+/-} and Gas6^{-/-} mice are viable, fertile, appear normal and show no obvious differences in size, weight or behaviour. No genotypic differences in litter size were observed. These mice appeared to be protected against thrombosis because of a platelet dysfunction (Angelillo-Scherrer *et al.* (2001) *Nat Med* **7**, 215-21).

Gas6 expression has been detected in hematopoietic tissue, both in hematopoietic (megakaryocytes and myelomonocytic precursors) and stromal (endothelial cells, fibroblasts, adipocytes) cells (Avanzi *et al.* (1997) *Exp Hematol* **25**, 1219-26). Gas6 receptor Axl is expressed in hematopoietic progenitors and bone marrow stromal cells, at low levels in monocytes, and in neoplastic cells of the myeloid lineage (Neubauer *et al.* (1994) *Blood* **84**, 1931-1941). Axl and Sky are detectable at sites of embryonic hematopoiesis (Crosier *et al.* (1996) *Exp Hematol* **24**, 318-323). However, Gas6 is considered to have no mitogenic effect in the hematopoietic system, and not affecting the proliferative stimuli exerted by other hematopoietic growth factors (Avanzi *et al.* (1997), cited above). More recently, monolayers of Gas6 expressing fibroblasts were shown to support the generation of colony-forming units in culture. This hematopoietic support is not vitamin K-dependent and soluble recombinant

Gas6 does not substitute for co-culturing the hematopoietic progenitors with Gas6 expressing fibroblasts (Dormady *et al.* (2000) *Proc Natl Acad Sci U S A.* **97**, 12260-12265).

WO 96/28548 describes Gas6 as the activator of the Rse and Mer receptor protein kinases and suggests the use of Gas6 for the enhancement of survival, proliferation or differentiation of any cell type expressing these proteins.

Summary of the invention

10 An object of the present invention is to provide formulations and methods for the treatment or prevention or cure of anemia in humans or mammals and for the manufacture of a medicament to treat a human or mammalian patient to prevent, reduce or cure anemia of said patient. In particular, the anemia can be induced by hemolysis or caused by or associated with various disorders such
15 as chronic disease, chronic renal failure, aplastic anemia, hemolytic anemia, malignancies, endocrine deficiencies or can be caused by a specific treatment such as chemotherapy.

The present invention is based on a first observation that growth arrest-specific gene 6 (Gas6) expression is required for the development of sufficient erythroid reserves and to ensure an adequate hematopoietic response to an
20 anemic challenge in humans and/or mammals. Furthermore it was found that treatment with a Gas6 compound can provide protection against anemia induced by hemolysis.

The present invention relates to a new method for the treatment of anemia based on the administration of a Gas6 product, e.g. the gene product of
25 the growth arrest-specific gene 6 (Gas6), its mutants, variants, active derivatives and the physiological tolerated salts of these Gas6 derivatives or of a group of biologically active substances inducing a Gas6 expression or releasing action or of a group of compounds that activate the Sky, Axl and/or
30 Mer receptor tyrosine kinases.

Thus, one object of present invention is the use of a Gas6 compound, e.g. Gas6 protein or an analogue, mutant, variant or derivative thereof, or a

physiologically tolerated salt of said Gas6 derivative for the treatment or prevention of anemia and for the manufacture of a medicament to treat a patient to prevent, reduce or cure anemia of said patient. Another object is the use of biologically active substances inducing a Gas6 expression or release for the treatment or prevention of anemia and for the manufacture of a medicament for the prevention or treatment of anemia. Biologically active substances supporting, provoking or inducing a Gas6 expression can be identified by several methods. On example is a vector comprising the Gas6 gene or its polymorphisms (with or without promoters) that can be vectored into relevant cells. Such vectors may include plasmids or viral vectors for example as well known from gene therapy. Another example is a monoclonal antibody which binds to the Axl and/or Tyr and/or Mer receptor and activates these. Immunological techniques or PCR can be used to detect increased expression of Gas6 in cell cultures or in test animals. Alternatively, reporter constructs comprising the Gas6 promoter region operably linked to a reporter gene (e.g. luciferase, GFP, LacZ or Chloramphenicol transferase) can be used for screening transfected cells or transfected test animals such as zebrafish or *C. elegans*.

The anemia can be caused by or associated with various disorders such as chronic disease, chronic renal failure, aplastic anemia, hemolytic anemia, malignancies, endocrine deficiencies or can be caused by a specific treatment such as chemotherapy.

An additional observation in the context of the present invention was that administration of a Gas6 compound, in dosages which provided the protective effect against anemia did not result in above-average hematocrit levels or other side-effects, such as polycythemia. The results obtained by the present inventors indicate that treatment with a Gas6 compound can correct anemia, but will do so only up to levels that are in the normal range, not leading to excessive hemoglobin levels as observed with Epo. This is an important advantage for all applications of a Gas6 compound in the treatment of anemia, but may be a critical factor for the treatment of anemia in patients susceptible to the adverse side-effects of Epo, such as hypertension or polycythemia.

Thus, a further object of the present invention is the use of a Gas6 compound, e.g. Gas6 protein or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative for the treatment or prevention of anemia in patients for which treatment with Epo (or
5 an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) is contra-indicated and for the manufacture of a medicament for the prevention or treatment of anemia in patients for which treatment with Epo is contra-indicated.

The invention is furthermore based on the observation that in the
10 absence of Gas6, response to Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) is sub-optimal and that administration of a Gas6 compound together with erythropoietin or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative, results in a synergistic
15 rescue effect on erythropoiesis. This indicates that that the level of Gas6 can be considered as a limiting factor for the effectiveness of Epo. Moreover, the present results indicate that upon combination of a Gas6 compound and Epo or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative, the dose of Epo can be reduced to levels which
20 would normally be considered as sub-optimal, thereby diminishing the likelihood of the development of adverse effects related to Epo.

Thus, a further object of the invention is the use of a Gas6 compound such as Gas6 protein or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative for the treatment or
25 prevention of anemia in patients non-responsive to Epo or for the manufacture of a medicament for the treatment or prevention of anemia in a patient non-responsive to Epo.

A further object of the present invention is the use of a Gas6 compound such as Gas6 protein or an analogue, mutant or derivative thereof, or a
30 physiological tolerated salt of said Gas6 derivative, in combination with erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) for the treatment or

prevention of anemia or for the manufacture of an antianemic drug or an antianemic composition. These medicaments can be used to increase the survival rate of the patient suffering of anemia.

The invention further relates to a method of selection of a formulation of
5 a pharmaceutical ratio of a Gas6 compound to Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative), said method comprising: determining the minimal dose of Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) which does not induce adverse side-effects in a patient;
10 and administering in addition to a) incremental doses of the Gas6 compound so as to determine which combined Epo/Gas6 compound dosage ensures maintenance of target hemoglobin levels in said patient, selecting the ratio in accordance with the determination of step b)

Alternatively, according to the present invention a method of selection of
15 a formulation of a pharmaceutical ratio of a Gas6 compound to Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative), is provided wherein said method comprises: determining independently, the minimal dose of Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO
20 derivative) and Gas6 compound which ensures elevation of hemoglobin level to the target level in a patient, and administering a combination of the minimal dose of both Epo (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) and Gas6 compound and gradually reducing the dosage of Epo (or an analogue, mutant, variant or
25 derivative thereof, or a physiologically tolerated salt of said EPO derivative), so as to determine which combined Epo/Gas6 compound dosage ensures maintenance of target hemoglobin levels in said patient, without inducing the adverse side-effects of Epo.

A particular embodiment of this aspect of the invention relates to the use
30 of a Gas6 compound, such as Gas6 protein or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative in an optimized dosage combination with Epo (or an analogue, mutant, variant or

derivative thereof, or a physiologically tolerated salt of said Epo derivative) for the treatment or prevention of anemia in a patient for which treatment with Epo is contra-indicated, or in a patient non-responsive to Epo. Similarly, the invention relates to the use of a Gas6 compound such as Gas6 protein or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative for the manufacture of a medicament for the prevention or treatment of anemia in a patient for which treatment with Epo is contra-indicated, or in a patient non-responsive to Epo.

The invention further relates to method of augmenting the anti-anemic effect of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) being administered to a patient being afflicted with anemia, said method comprising administering to said patient an erythropoietin anti-anemia effect augmenting amount of a Gas6 compound in combination with a portion of an effective amount of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) normally required to treat anemia in said patient in the absence of said Gas6 compound, thereby decreasing the dosage of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) normally required to treat anemia in said patient to said portion of an effective amount and reducing the side effects caused by erythropoietin.

Optionally, the invention relates to the above-described method, wherein the amount of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) being administered is 5 to 90% of the amount of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative) normally administered in the absence of said Gas6 compound.

Accordingly, a method is provided for the treatment, curing or prevention of anemia in a patient for which treatment with Epo is contra-indicated or in a patient who is irresponsive to Epo, which comprises administering thereto an optimized combination of a Gas6 compound such as Gas6 protein or an analogue, mutant or derivative thereof, or a physiologically tolerated salt of said

Gas6 derivative, and erythropoietin, either simultaneously or sequentially.

According to another aspect of the present invention a method is provided for the treatment, curing or prevention of anemia, wherein the patient to be treated receives an effective amount biologically active substances inducing a Gas6 expression or releasing action and for a suitable time to prevent, reduce or cure anemia. This method may further comprise administering of erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative).

Yet another embodiment is an anti-anemic pharmaceutical composition comprising a Gas6 compound such as Gas6 protein or an analogue, mutant or derivative thereof, or a physiological tolerated salt of said Gas6 derivative or an optimized formulation of such a Gas6 compound with erythropoietin (or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative).

Description of the illustrative embodiments

The reference to Gas6, a mutant, variant or derivative thereof (generally referred to herein as Gas6 compounds) includes the Gas6 protein as encoded by the human Gas6 gene (Manfiotti *et al.* (1993) *Molec. Cell Biol* 13, 4976-4985), any proteins having a modified amino acid sequence, whereby this modification does not detrimentally affect the activity of Gas6 described herein. Reference to Gas6 or a Gas6 compound excludes EPO or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative. The reference to Gas6 therefore includes species (human or animal) specific homologs or orthologs of Gas6 as well as truncations, deletions, point mutations, substitutions or other modifications made by man which maintain functionality. The maintenance of the activity of a Gas6 protein with a modified amino acid can be compared by determining for example the changes in hematocrite level of such modified protein, when compared to the wild type protein. Alternatively, reference to Gas6 function of a Gas6 compound may include proteins having a modified amino acid sequence, whereby this modification does not affect the activation of the Tyr and/or Mer and/or Axl

receptor, the activation being of the type that is induced by wildtype Gas6. Activation of the Tyr or Mer receptor can be tested by assaying the tyrosine phosphorylation by Tyr or Mer of a substrate peptide. As used herein "mutants of Gas6" refers to modified Gas6 proteins of equal length as wild type Gas6 but
5 with one or more modified amino acids. The sequence homology of such modified Gas6 proteins have a homology of 95% or greater, or 98% or greater or 99% or greater compared to the wildtype Gas6. As used herein "variants" refers to modified Gas6 proteins with a length differing from wild type Gas6 obtained by removal or addition of one or more amino acids (either N-terminal,
10 C-terminal or internally, for instance the variants which are less or not γ -carboxylated, fragments of Gas6 which lack the A domain as well as fragments which consist essentially of the D domain, such as those described in WO 96/28548. The sequence identity of such variant Gas6 proteins can be 70% or greater compared to the wildtype Gas6.

15 A Gas6 analogue as used herein refers to a molecule capable of activating the Tyr, Mer and/or Axl receptor on hematopoietic cells, in a similar way as Gas6. These molecules can optionally be directly or indirectly derived from Gas6. Protein S has been reported to be a ligand for Tyro3, which is a receptor of the Tyr, Mer and Axl family and which binds also Gas6. Gas6
20 analogues can have a structure being related to Gas6 (peptide analogues) or unrelated to Gas6 such as small compounds. Gas6 analogues can be screened by evaluating the activation of a Gas6 receptor by assaying for example the tyrosine kinase activity of such receptor on a substrate peptide.

A physiologically tolerated salt of Gas6, or a mutant, variant or derivative
25 thereof refers to any therapeutically active non-toxic salts which the Gas6 compounds are able to form with a salt-forming agent. Such addition salts may conveniently be obtained by treating the Gas6 compounds of the invention with an appropriate salt-forming acid or base. Examples of such appropriate salt-forming acids include, for instance, inorganic acids resulting in forming salts
30 such as but not limited to the hydrochloride, hydrobromide, sulfate, nitrate, phosphate, diphosphate, bicarbonate, carbonate salts, and the like; or organic monocarboxylic or dicarboxylic acids resulting in forming salts such as, for

example, the acetate, propanoate, hydroxyacetate, 2-hydroxypropanoate, 2-oxopropanoate, lactate, pyruvate, oxalate, malonate, succinate, maleate, fumarate, malate, tartrate, citrate, methanesulfonate, ethanesulfonate, benzoate, benzenesulfonate, p-toluene-sulfonate, salicylate, p-aminosalicylate, pamoate, bitartrate, camphorsulfonate, edetate, 1,2-ethanedisulfonate, fumarate, glucoheptonate, gluconate, glutamate, hexylresorcinate, hydroxynaphthoate, hydroxyethanesulfonate, lactate, mandelate, methylsulfate, pantothenate, stearate and the like. Examples of appropriate salt-forming bases include, for instance, inorganic bases like metallic hydroxides such as but not limited to those of calcium, lithium, magnesium, potassium, sodium and zinc; organic bases such as but not limited to N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, ethylene-diamine, N-methylglucamine, procaine and the like.

Erythropoietin or Epo as used herein refers to the naturally occurring human cytokine, produced primarily in the kidney which stimulates the production of red blood cells, as well as analogues, mutants, variants or derivatives thereof, or a physiologically tolerated salt of said EPO derivative. Well described erythropoietin analogues are the hyperglycosylated recombinant proteins epoetin and darbepoetin (or novel erythropoiesis stimulating protein, NESP), of which the structures differ from naturally occurring Epo only by the number of N-linked oligosaccharide on the protein. The invention however also envisages the possible development of other activators of the Epo receptor, which if developed into Epo analogues, can be used in the context of the present invention. The expression product of deletion mutants of a synthetic human Epo cDNA, wherein the point mutations and small deletions in helices and interhelical regions of the four alpha helical bundle motif have been shown to display biological activity (Bittorf *et al.* (1993) *FEBS Lett* **336**, 133-136; Boissel *et al.* (1993) *J. Biol. Chem.* **268**, 15983-15993). Furthermore, the screening of combinatorial libraries of dimeric iminodiacetic acid diamides, small molecule binders of the Epo receptor have been identified, of which some were found to be partial agonists (Goldberg *et al.* (2002) *J Am Chem Soc* **124**, 544-555) In addition Johnson *et al* (1998) *Biochemistry* **37**, 3699-3710 present

amino acid peptide mimetics of EPO which were selected by phage display. US patent application US2003/0113871 discloses EPO fusion proteins with increased half-life. US patent application 2003/0077753 describes EPO variants with modified glycosylation patterns.

- 5 Anemia as used herein refers to a condition in which the number of red blood cells per mm^3 , the amount of hemoglobin in 100 ml blood and/or the volume of packed red blood cells per 100 ml blood are less than normal. When observing hemoglobin levels, a level of less than 9 g/dl is usually considered as indicative of anemia, though individual variations should be taken into account
- 10 (see below). Potential causes include blood-loss, nutritional deficits, medication reaction, various problems with the bone marrow and many diseases. More particularly, anemia has been associated with a variety of disorders that include, for example, chronic renal failure, myelodysplastic syndrome, rheumatoid arthritis, and bone marrow transplantation. It will be understood that in the
- 15 context of the present invention, the anemic conditions susceptible to treatment with Gas6 optionally in combination with Epo exclude those conditions where anemia can be attributed to the absence of nutrients, such as vitamin B12 deficiency. More particularly, the use of Gas6, optionally in combination with Epo is envisaged in the treatment and/or prevention of the following disorders and conditions:
- 20 - Anemia that is associated with malignant disease, including:
- solid tumors (e.g. breast cancer, lung cancer, colon cancer)
- tumors of the lymphatic system (e.g. chronic lymphocyte leukemia, non-Hodgkins and Hodgkins lymphomas)
- 25 - tumors of the hematopoietic system (eg. leukemia, myelodysplastic syndrome, multiple myeloma).
- Anemia associated with radiation therapy.
- Anemia associated with chemotherapy (e.g. platinum containing regimens) for cancers.
- 30 - Anemia associated with inflammatory and autoimmune diseases, including, but not limited to, rheumatoid arthritis, other inflammatory arthritides, systemic lupus (SLE), acute or chronic skin diseases (e.g. psoriasis), inflammatory bowel

disease (e.g. Crohn's and Ulcerative Colitis).

- Anemia associated with acute or chronic renal disease or failure due to any cause, including those not defined, i.e. idiopathic or congenital.
 - Anemia associated with acute or chronic liver disease.
 - 5 - Anemia associated with acute or chronic bleeding.
 - Anemia for any reason where transfusion of red blood cells is not possible due to patient allo- or auto-antibodies and/or for religious reasons (e.g. some Jehovah's Witnesses).
 - Anemia associated with infections (e.g. malaria, osteomyelitis)
 - 10 - Anemia associated with hemoglobinopathies, including, for example, sickle cell disease, thalassemias.
 - Anemia associated with any chronic disease.
 - Anemia associated with drug use or abuse, e.g. alcohol misuse.
 - Pediatric patients with anemia from any cause to avoid transfusion.
 - 15 - Elderly patients or patients with underlying cardiopulmonary disease with anemia who cannot receive transfusions due to concerns about circulatory overload.
 - Patients who are iron-overloaded, yet require raised hemoglobin. These would include all patients with ineffective erythropoiesis of any cause.
- 20 The 'target hemoglobin level' as referred to herein is considered a hemoglobin level of between about 10 g/dl and about 12.5 g/dl preferably about 11.0 g/dl (see also Jacobs *et al.* (2000) *Nephrol Dial Transplant* **15**, 15-19). Hemoglobin levels in healthy individuals ('normal hemoglobin level') are usually significantly higher (14-17.4 g/dl for males and 12.3-15.3 g/dl for females;
- 25 Wintrobe MM, Clinical hematology, 10th Ed Philadelphia.), without necessarily resulting in any clinical symptoms. Alternatively, hematocrit levels (percentage of the volume of a blood sample occupied by the cells, can be used as a measure for the condition of red blood cells. Hematocrit levels for healthy individuals range from 41 to 51 for adult males and from 35 to 45 for adult
- 30 females. Target hematocrit levels are usually around 30-33%. It has been reported that upon obtaining a normal Ht ($42.0 \pm 1.9\%$) in hemodialysis patients, both physiological tests and quality of life improved significantly without adverse

effects (Minetti (1997) *J Nephrol* 10, 117-119). Thus, in the absence of contra-
indications (e.g. high cardiovascular risk), higher hemoglobin/hematocrit target
levels can be contemplated ('normalized hemoglobin level'), as long as adverse
effects are not observed or anticipated. Moreover, it is understood to the person
5 skilled in the art that hemoglobin/hematocrit levels vary from person to person.
Thus, optimally, the target hemoglobin/hematocrit level should be individualized
for each patient (Jacobs *et al.*, cited above). 'Overshooting' or an excessive
hemoglobin level as used herein, refers in a situation whereby a level of
hemoglobin above 13 g/dL or higher than the normalized hemoglobin level is
10 associated with adverse side-effects, such as hypertension and/or
polycythemia.

A patient with an **"inadequate Epo response"** as used herein refers to
a patient in which administering of a normal to increased (>300IU/kg/week)
dose of Epo does not result in the increase of haemoglobin level up to the target
15 level. Optionally, in patients susceptible to adverse effects of Epo, the absence
of a response to standard dosage of Epo (around 50-100U/kg weekly) can be
considered as **"inadequate response to a tolerable dose of Epo"**. Patients
with an inadequate Epo response are found for all types of anemia, but higher
numbers of non-responders have been observed particularly frequently in
20 patients with cancers and patients with end-stage renal disease.

An inadequate response to Epo can be either constitutive (i.e. observed
upon the first treatment with Epo) or acquired (e.g. observed upon repeated
treatment with Epo). Thus, patients who do not show an initial positive response
(i.e. reaching of target hemoglobin level) to Epo are considered **"irresponsive
25 to treatment with Epo"** in the context of the present invention.

When an initial response to treatment with Epo is observed, but the
response **"resistant to Epo"** refers to patients in which, upon prolonged and/or
repeated treatment with Epo, there is a decreased response to exogenous Epo.
This resistance has been linked to iron or folate deficiency, aluminum toxicity,
30 hyperparathyroidism or auto-antibodies. In the case of an immune reaction to
exogenous Epo the immune reaction can sometimes be overcome with anti-
CD4+ mAB administration (Rinsch *et al.* (2002) *Kidney Intern.* **62**, 1395-1401).

A subset of patients with an inadequate Epo response is formed by those patients for which an elevated endogenous Epo level is observed. The absolute concentration of Epo considered as **"elevated"** is dependent on the disease and dosage of Epo. It has been observed that in Epo treatment of anemia associated with HIV infection and zidovudine therapy (100-200U/kg, three times a week for up to 12 weeks), patients with Epo levels greater than 500IU/L showed no benefit from recombinant Epo.

An **"adverse effect of Epo"** as used herein refers to effects which are associated with the Epo treatment and are unwanted. The primary adverse effects of Epo are an excessive increase in the hematocrit or hemoglobin levels and polycythemia. Elevated hematocrit levels can lead to hypertension (more particularly aggravation of hypertension) and vascular thrombosis. Other adverse effects of Epo which have been reported, some of which related to hypertension, are headaches, influenza-like syndrome, obstruction of shunts, myocardial infarctions and cerebral convulsions due to thrombosis, hypertensive encephalopathy, and red cell blood cell aplasia (Singlbartl, (1994) *J. Clin Invest* **72(suppl 6)**, S36-S43; Hörl *et al.* (2000) *Nephrol Dial Transplant* **15(suppl 4)**, 51-56; Delanty *et al.* (1997) *Neurology* **49**, 686-689; Bunn (2002) *N Engl J Med* **346(7)**, 522-523).

A patient **"susceptible to the adverse effect of Epo"** or **"for which treatment with Epo is contra-indicated"** as used herein refers to a patient for whom, due to natural (i.e. genetic) predisposition or due to other factors (e.g. disease, medication etc.), the adverse side effects of Epo would be more detrimental than for a person not having this natural or unnatural predisposition. More particularly, patients with increased blood pressure, either as a result of a genetic predisposition or as a result of vascular disease or medication, are considered more susceptible to the aggravation of hypertension observed in the treatment with Epo.

The present invention relates to the treatment and/or prevention of anemia with an effective amount of a Gas6 compound. An effective amount of a Gas6 compound to be employed therapeutically will depend, for example, on the therapeutic objections, the route of administration, and the condition of the

patient. Accordingly it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. The effective amount is usually in the range of 0.001mg to 100 mg, preferably 0.1 mg to 5 mg, per day per kg bodyweight for humans. Depending upon the pathologic condition to be treated and the patient's condition, the said effective amount may be divided into several sub-units per day or may be administered at more than one-day intervals.

Where possible, it is desirable to determine appropriate dosage ranges first *in vitro*, for example, using assays for cell survival or growth, which are known in the art, and then in suitable animal models, from which dosage ranges for human patients may be extrapolated. In a specific embodiment of the invention, a pharmaceutical composition effective in stimulating hemoglobin production by hematopoietic cells will provide a local gas6 concentration *in vivo* of between about 0.1 and 10 ng/ml.

Alternatively, delivery of Gas6 and/or erythropoietin in the context of the present invention can be performed using gene therapy. Therapeutic levels of Epo using gene therapy has been described in the art, including in WO 95/13376 and EP 1105506. Similar technology can be applied for the administration of Gas6. Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a Gas6 polypeptide, a mutant, variant or derivative thereof or a Gas6 analogue *ex vivo*, with the engineered cells then being returned to said patient. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art using a retroviral or lentiviral particle containing RNA encoding a polypeptide of the present invention. Cells suitable for such cell-based gene therapy include, but are not limited to stem cells, sertoli cells, and include the use of encapsulated xenogeic cells. Alternatively, vectors (such as infectious viral particles or non-viral particles such as liposomes or micelles) comprising a DNA encoding a Gas6 polypeptide, a mutant, variant or derivative thereof or a Gas6 analogue, can be used for the engineering of Gas6 expressing cells *in vivo*.

The present invention further relates to the treatment and/or prevention

of anemia with an optimized combination of Gas6 and Epo. Establishing an optimal combination of Epo and Gas6 requires titration of the two components, each adjusted to achieve a normal hemoglobin level, without "overshooting" the normal or target range, followed by a combined treatment with increasingly
5 reduced dosage, so as to determine which minimal dosage can ensure the target or normalized hemoglobin level without excessive hemoglobin production and without adverse side effects.

The optimal combination of Gas6 and Epo can optionally be determined for every individual patient, so as to take into account individual susceptibility to
10 adverse effects of Epo or Gas6 and/or individual irresponsiveness to Epo and/or Gas6.

The pharmaceutical compositions and combined preparations according to this invention may be administered orally or in any other suitable fashion. Oral administration is preferred and the preparation may have the form of a
15 tablet, aqueous dispersion, dispersable powder or granule, emulsion, hard or soft capsule, syrup, elixir or gel. The dosing forms may be prepared using any method known in the art for manufacturing these pharmaceutical compositions and may comprise as additives sweeteners, flavouring agents, colouring agents, preservatives and the like. Carrier materials and excipients are detailed
20 hereinbelow and may include, *inter alia*, calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, binding agents and the like. The pharmaceutical composition or combined preparation may be included in a gelatine capsule mixed with any inert solid diluent or carrier material, or has the form of a soft gelatine capsule,
25 in which the ingredient is mixed with a water or oil medium. Aqueous dispersions may comprise the biologically active composition or combined preparation in combination with a suspending agent, dispersing agent or wetting agent. Oil dispersions may comprise suspending agents such as a vegetable oil. Rectal administration is also applicable, for instance in the form of
30 suppositories or gels. Injection is also applicable as a mode of administration, for instance in the form of injectable solutions or dispersions.

The term "**pharmaceutically acceptable carrier or excipient**" as used

herein in relation to pharmaceutical compositions and combined preparations means any material or substance with which the biologically-active ingredient(s), i.e. the Gas6, or an analogue, mutant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative, may be formulated in order to facilitate its application or dissemination to the locus to be treated, for instance by dissolving, dispersing or diffusing the said composition, and/or to facilitate its storage, transport or handling without impairing its effectiveness. The pharmaceutically acceptable carrier may be a solid or a liquid or a gas which has been compressed to form a liquid, i.e. the compositions of this invention can suitably be used as concentrates, emulsions, solutions, granulates, dusts, sprays, aerosols, pellets or powders. Suitable pharmaceutical carriers for use in the said pharmaceutical compositions and their formulation are well known to those skilled in the art.

The present invention will be demonstrated in more detail in the following examples, which are however not intended to limit the scope of the invention, and may be understood in conjunction with the accompanying Figures, which show:

Figure 1: a,b, Reduced number of erythroid progenitors in fetal liver of Gas6^{-/-} mice, analyzed as the number of BFU-E (n = 6; mean ± S.E.M.; *, P < 0.05; panel a) or CFU-E (n = 6; mean ± S.E.M.; *, P < 0.05; panel b). c,d, Reduced percentage of Ter-119⁺ nucleated cells (erythroblasts) in adult bone marrow (n = 9; mean ± S.E.M.; *, P = 0.02) and spleen (n = 9; mean ± S.E.M.; *, P < 0.02) in Gas6^{-/-} mice. The erythroblasts were quantified as % of all nucleated cells by flow cytometry. e,f, Immunohistochemistry of adult WT (e) and Gas6^{-/-} (f) spleen, showing fewer brown-stained Ter-119⁺ cells in Gas6^{-/-} mice. g,h, Reduced number of erythroid progenitors in the bone marrow of adult Gas6^{-/-} mice, analyzed as the number of BFU-E (n = 8; mean ± S.E.M.; *, P < 0.05; panel g) or CFU-E (n = 8; mean ± S.E.M.; *, P < 0.05; panel h).

Figure 2: a,b, Impaired erythropoiesis in Gas6^{-/-} mice in response to phenylhydrazine (PHZ) induced hemolytic anemia. c,d, Impaired

erythropoiesis in Gas6^{-/-} mice in response to autoimmune hemolytic anemia, induced by intraperitoneal injection of 34-3C anti-mouse red blood cell monoclonal antibody (200 µg on day 0). Hematocrit (a,c) and reticulocyte index (b,d) levels are expressed as mean ± SEM (n = 8; *, P < 0.05).

Figure 3: Impaired Epo-survival response of Gas6^{-/-} erythroblasts. a, TUNEL assay of WT and Gas6^{-/-} nucleated spleen cells on day 6 after PHZ injection. Cells were cultured for 16 h in the presence or absence of recombinant human erythropoietin (Epo) at the indicated concentrations. TUNEL assay was quantified by flow cytometry (n = 8, mean ± SEM; *, p<0.05 versus +/+) and results expressed as percent of cells double-stained for TUNEL and Ter-119. b, Dead cell count of nucleated spleen cells (Trypan-blue positive cells) collected on day 6 after PHZ injection and maintained in culture for 24 h. in the presence or absence of Epo (1.5 IU/ml), or Epo plus recombinant human Gas6 (rGas6, 400 ng/ml) (n = 8, mean ± SEM; *, P < 0.05 versus +/+)

Figure 4: Gas6 prevents anemia induced by acute hemolysis or blood loss. a,b, Wild type mice (a) and Gas6^{-/-} mice (b) were subjected to PHZ induced hemolytic anemia and treated with saline (control), recombinant human Gas6 (rGas6, 2 µg daily intraperitoneally), recombinant human erythropoietin (Epo, 10 IU every second day intraperitoneally) or a combination of rGas6 and Epo. c, After bleeding (500 µl) on day 0 and 1, wild type mice were treated with saline, Epo, rGas6 or Epo + rGas6 as above, and the erythropoietic response was monitored by determining the hematocrit levels. Hematocrit levels are expressed as mean ± SEM (n = 6 mice) in all panels.

Figure 5: Effect on hematocrit levels of erythropoietin deficient mice (143.LC, *Epo-Tag^H*) after administration of EPO alone (10 IU/day) or EPO in combination with recombinant Gas6 (EPO: 10 IU/day, rGas6 2 microgram/day). (Filled bars: day 0; striped bars: day 4)

EXAMPLE I: MICE DEFICIENT IN GAS6 (GAS6^{-/-} MICE) HAVE A DECREASED RETICULOCYTE COUNT

Animal experiments were conducted according to the guiding principles
5 of the American Physiological Society and the International Committee on
Thrombosis and Haemostasis (Giles *et al.* (1987) *Thromb Haemost* **58**, 1078-
84).

Blood was collected under general anesthesia from retrobulbar plexus of
wild type mice (Gas6^{+/+} mice) or mice in which Gas6 expression was abolished
10 by homologous recombination (Gas6^{-/-} mice) (Angelillo-Scherrer *et al.* (2001),
cited above). Reticulocyte counts were performed on smears of blood that had
been stained with New Methylene Blue according to standard protocol (Sigma
R4132). At least 1000 red blood cells were counted in each determination.

The data are represented as mean \pm SEM of n determinations. The
15 significance of differences was determined by unpaired Students'-test.
Reticulocytes represented 24.6 ± 4.2 ‰ (n=8) of the circulating red blood cells
in Gas6^{+/+} mice and only 13.1 ± 3.1 ‰ (n=8) of the red blood cells in Gas6^{-/-}
mice (p<0.001). These data indicate that Gas6 is necessary for maintaining the
reticulocyte count within the normal range.

EXAMPLE II: GAS6 DEFICIENT (GAS6^{-/-}) MICE HAVE REDUCED ERYTHROID RESERVES

Single-cell suspensions were obtained from bone marrow and spleen
isolated from Gas6^{+/+} and Gas6^{-/-} mice. Ammonium chloride lysis of mature red
25 blood cells was performed. Bone marrow and spleen cells (10^6) were incubated
on ice with rat anti-mouse CD16/CD32 to block nonspecific binding to Fc
receptors. Cell suspension was then stained with (PE)-conjugated-anti-Ter-119
antibody (PharMingen) for 30 min. at 4°C in 100 μ l phosphate-buffered saline
containing 0.2% BSA. Appropriate isotype control antibodies were used. Cell
30 surface expression of Ter-119 was analyzed in a Becton Dickinson FACScan
using CellQuest software.

Ter-119 is expressed by cells of the erythroid lineage, from

proerythroblast to mature red blood cells. Ter-119 is expressed by 29 ± 3 % (mean \pm SEM, n=3) of Gas6^{+/+} bone marrow cells versus 17 ± 3 % (mean \pm SEM, n=3) of Gas6^{-/-} bone marrow cells ($p < 0.05$). Similarly, Ter-119 is expressed by 6.0 ± 1.3 % (mean \pm SEM, n=3) of Gas6^{+/+} spleen cells versus 1.9 ± 0.5 % (mean \pm SEM, n=3) of Gas6^{-/-} spleen cells ($p < 0.05$).

EXAMPLE III: THE NUMBER OF ERYTHROID PROGENITORS IS DECREASED IN THE BONE MARROW OF MICE DEFICIENT IN GAS6 (GAS6^{-/-} MICE)

In vitro clonogenic assays for progenitor cells were used to study distinct populations at different stages of development. Single-cell suspensions were prepared from bone marrow and spleen of adult mice or livers of day 13.5 embryos (E13.5) and counted in the presence of 3% acetic acid to lyse erythrocytes. For this, cell suspensions were mixed with MethoCult M3434 (StemCell Technologies, Vancouver) as described (Neubauer *et al.* (1998) *Cell* **93**, 397-409). Cells were plated in 35 mm dishes and cultured at 37°C, 5% CO₂. Colonies, including erythroid burst or blast-forming units (BFU-E, early erythroid progenitor), were scored at day 7. For the final progenitor cell erythroid colony-forming units (CFU-E) assay, cells were cultured in MethoCult 3230 containing 0.2 U/ml recombinant murine erythropoietin (R&D Systems) and colonies were scored at day 3.

Reduced erythroid progenitor cells in Gas6^{-/-} embryos

Upon macroscopic inspection, Gas6^{-/-} embryos did not appear paler than WT embryos, indicating that they were not severely anemic. Microscopic examination of E13.5 fetal liver sections showed a similar fraction of erythroid cells per section in both genotypes. This was confirmed by flow cytometry, in which the number of Ter-119⁺ erythroblasts per nucleated liver cell was not significantly different in homogenates of fetal livers from Gas6^{-/-} and WT mice. However, the absolute number of nucleated cells per liver was significantly decreased as compared to the livers of E13.5 wild-type (WT) embryos ($5.5 \pm 0.9 \times 10^6$ versus $11.9 \pm 2.8 \times 10^6$ nucleated liver cells/liver in Gas6^{-/-} and WT

embryos, respectively; $n = 6$; $P < 0.002$).

To examine why the erythroid reserve, reflected by the absolute number of fetal liver erythroblasts, is reduced in $\text{Gas6}^{-/-}$ embryos, erythroid progenitor cells were quantified in the fetal liver, using *in vitro* clonogenic assays. The number of E13.5 fetal liver erythroid blast-forming units (BFU-E) and erythroid colony-forming units (CFU-E), relative to the total number of nucleated liver cells, was similar in both genotypes (187 ± 12 BFU-E/ 10^5 fetal liver cells and 303 ± 50 CFU-E/ 10^5 fetal liver cells in WT versus 211 ± 18 BFU-E/ 10^5 fetal liver cells and 307 ± 64 CFU-E/ 10^5 fetal liver cells in $\text{Gas6}^{-/-}$; $n = 6$; $P = \text{n.s.}$). However, in absolute terms, $\text{Gas6}^{-/-}$ fetal livers contained only half the number of BFU-E and CFU-E as compared to WT fetal livers (Figure 1a,b). Thus, in the absence of Gas6, the diminished embryonic erythroid reserve may be accounted for by a reduction in erythroid progenitors.

15 **Reduced erythroid progenitor cells in $\text{Gas6}^{-/-}$ adult mice**

It was further examined whether erythroid reserves were also reduced in adult $\text{Gas6}^{-/-}$ mice. Hematocrit levels, red blood cell indices and the morphology of peripheral blood and bone marrow erythroid cells were all normal in $\text{Gas6}^{-/-}$ mice (hematocrit: $48.8 \pm 1.0\%$ in WT mice versus $49.1 \pm 3.9\%$ in $\text{Gas6}^{-/-}$ mice; $n = 12$; $P = \text{n.s.}$). However, in $\text{Gas6}^{-/-}$ mice, the rate of new red blood cell production was significantly impaired³³ (reticulocyte count: $2.5 \pm 0.1\%$ in WT mice versus $1.3 \pm 0.1\%$ in $\text{Gas6}^{-/-}$ mice; $n = 8$; $P < 0.002$). Clearance of *in vivo* biotinylated red blood cells was similar in both genotypes (percent biotinylated red blood cells after 30 days: $31 \pm 1.3\%$ in WT and $27 \pm 1\%$ in $\text{Gas6}^{-/-}$ mice, $n = 6$, $P = \text{n.s.}$).

Subsequently, erythroid progenitor cells in the bone marrow and spleen of adult mice were assessed. Compared to WT mice, adult $\text{Gas6}^{-/-}$ mice had significantly fewer erythroblasts in both their bone marrow and spleen (Figure 1c,d). The latter was also microscopically evident by immunostaining histologic sections of spleens with anti-Ter-119 antibody (Figure 1e,f). Additionally, the diminished number of erythroblasts in the spleens of $\text{Gas6}^{-/-}$ was paralleled by a reduction in the weight of spleens from $\text{Gas6}^{-/-}$ mice by ~20% (113 ± 3 mg in 33

WT mice versus 91 ± 15 mg in 31 Gas6^{-/-} mice; $P < 0.002$). To characterize the apparent defect in erythroblast proliferation/differentiation in Gas6^{-/-} mice, the number of distinct populations of erythroid progenitor cells in adult bone marrow was quantified. Three- to four-fold fewer colonies of BFU-E and CFU-E per 105
5 plated bone marrow cells developed from Gas6^{-/-} versus WT mice (Figure 1g,h). Thus, in the absence of Gas6, the number of erythroid progenitors was significantly reduced in the adult bone marrow.

10 **EXAMPLE IV: IMPAIRED RECOVERY AFTER ACUTE HEMOLYTIC ANEMIA OF GAS6 DEFICIENT MICE (GAS6^{-/-} MICE)**

Anemia was induced in Gas6^{+/+} and Gas6^{-/-} mice by intraperitoneal injection (0.5 or 2 mg /10 g body weight) of freshly prepared phenylhydrazine. Phenylhydrazine hydrochloride (Sigma P6926) was dissolved in PBS at either 10 or 20 mg/ml and the pH was adjusted to pH 7.4 with NaOH. At day 3
15 following treatment with low dose of PHZ (two doses of 0.5 mg/10 g, 8 hours apart), Gas6^{-/-} mice had a deeper depression in hematocrit (mean \pm SEM: 29 ± 0.5 %, $n=5$, $p<0.001$) than Gas6^{+/+} mice (hematocrit, mean \pm SEM: 36 ± 2 %, $n=5$). In addition, their spleen (site of red cell production) weighed less (mean \pm SEM: 142 ± 27 mg $n=4$, $p<0.05$) compared to Gas6^{+/+} mice (mean \pm SEM: 254
20 ± 28 mg, $n=4$), indicative of impaired erythropoiesis. Moreover, Gas6^{-/-} mice were more susceptible to hemolysis induced by a high dose (2 mg/10 g in one single dose) of PHZ as all Gas6^{-/-} ($n=10$) mice succumbed to the hemolysis, compared to the 25 % mortality rate in Gas6^{+/+} mice ($n=10$).

Taken together, these data indicate that lack of Gas6 expression
25 increases the susceptibility to acute hemolysis.

EXAMPLE V: GAS6^{-/-} MICE ARE RESISTANT TO EPO

Serum Epo levels in Gas6^{-/-} mice were measured in steady-state conditions and in response to anemia induced by PHZ. Under baseline
30 conditions, serum Epo protein levels were undetectable in both WT and Gas6^{-/-} mice (<20 mIU/ml). However, at days 3 and 6 after PHZ injection, serum Epo levels were significantly higher in Gas6^{-/-} than in WT mice (53 ± 12 mIU/ml in

WT versus 441 ± 120 mIU/ml in Gas6^{-/-} at day 3; 25 ± 0.5 mIU/ml in WT versus 165 ± 50 mIU/ml in Gas6^{-/-} at day 6, $n = 4-7$, $P < 0.05$). Epo receptor (EpoR) expression in spleen lysates was detected by Western immunoblot (Angelillo-Scherrer *et al.* (2001), cited above). In both WT and Gas6^{-/-} mice, EpoR was barely detectable under baseline conditions, but expression was dramatically increased 3 and 6 days after PHZ injection. While there was some variability, the EpoR levels between the two genotype mice were not significantly different. The data indicates that, in spite of elevated Epo levels and normal EpoR expression, there is an attenuated response to anemia in the Gas6^{-/-} mice. These results thus suggest that the Gas6-deficient mice are resistant to Epo and tried to compensate this defect – in vain however – by elevating their Epo production. Since iron reserves are necessary for a sufficient erythropoietic response and iron depletion is a common cause of resistance to Epo, the mice were assessed for iron stores. Perl's staining of bone marrow cytopins revealed adequate iron stores in both WT and Gas6^{-/-} mice (data not shown). Serum iron levels were also within the normal range (49 ± 2 μ m/l in WT and 33 ± 1.7 μ m/l in Gas6^{-/-}). Thus, the impaired erythropoietic response in anemic Gas6^{-/-} mice, in spite of elevated Epo levels, is not attributable to iron deficiency.

EXAMPLE VI: GAS6^{-/-} ERYTHROBLASTS ARE LESS SENSITIVE TO EPO AND UNDERGO INCREASED CELL DEATH

Spleens and livers were paraformaldehyde-fixed and 7 μ m paraffin sections were stained for Ter-119 (BD Biosciences Pharmingen, San Diego, CA). Apoptosis was evaluated by Terminal deoxyuridine triphosphate(TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin end (TUNEL)-labeling. ABC Vectastain kit (Vector, Burlingame, CA) with DAB-cobalt-nickel staining was used to localize TUNEL-positive cells.

TUNEL-staining of spleen sections from WT and Gas6^{-/-} mice under baseline conditions did not reveal any differences in the extent of apoptosis. However, 3 and 6 days after induction of hemolysis with PHZ, more apoptotic cells were detected in the red pulp of spleens from Gas6^{-/-} mice as compared to those from WT mice. To quantify these apparent differences, nucleated cells

were isolated from spleens 6 days after PHZ injection, when 80-90% of the nucleated cells are erythroblasts. When cultured for 16 h in the absence of Epo, approximately 50% of the erythroblasts were apoptotic, with no detectable difference between genotypes. However, the survival response provided by exogenously added recombinant Epo, was markedly different, depending on whether the erythroblasts were derived from WT or *Gas6*^{-/-} mice (Figure 4a). A concentration of 0.6 IU/ml of Epo decreased the number of apoptotic *Gas6*^{-/-} erythroblasts to 36 ± 2.1 % (from 51 ± 1.1 % when no Epo was added), which was significantly less protection afforded to WT erythroblasts, where this dose left only 25 ± 1.1 % apoptotic erythroblasts (down from 52 ± 0.7 % apoptotic cells with no Epo added). Indeed, a 2.5-fold higher concentration of Epo was required to provide *Gas6*^{-/-} erythroblasts similar protection against apoptosis. These findings were further confirmed with Trypan blue staining to assess erythroblast death, showing that the Epo-survival response was impaired in the absence of Gas6. Finally, addition of recombinant Gas6 (rGas6) with exogenous Epo significantly reduced the death of WT and *Gas6*^{-/-} erythroblasts, indicating that rGas6 fully restored the impaired Epo-survival response of *Gas6*^{-/-} erythroblasts. These data indicate that lack of Gas6 in erythroblasts prevents an optimal survival response to Epo, and that exogenous soluble rGas6 may rescue the cells from death.

Gas6, Tyro 3, Axl and Mer expression in erythroblasts

Assessment of the expression in the spleen of Gas6 receptors was performed under baseline conditions and 6 days after PHZ-induced hemolytic anemia when 80-90% of the splenic cells are erythroblasts (Angelillo-Scherrer *et al.* (2001), cited above). Tyro 3, Axl and Mer expression were all detectable by Western immunoblot, and without significant differences in intensity between WT and *Gas6*^{-/-} mice (Figure 3b). UT7 cells, a human erythroid cell line, were treated with Epo (Figure 3c). Gas6 was readily detected by Western immunoblot in lysates of quiescent cells. Both cellular and secreted Gas6 expression were augmented by Epo in a dose-dependent manner, providing additional support for a model in which Epo may upregulate and induce the

secretion of Gas6.

EXAMPLE VII: GAS6 PREVENTS ANEMIA INDUCED BY HEMOLYSIS OR BLOOD LOSS

5 WT and Gas6^{-/-} mice were injected on days 0 and 1 with PHZ, and treated from day 0 with saline i.p. (controls) daily, rGas6 2 µg i.p. daily, recombinant Epo (rEpo) 10 IU i.p. every second day, or both rGas6 and rEpo. Hematocrits and reticulocyte counts were monitored. Since rGas6 has mitogenic and survival effects on several cell types, it was first confirmed that
10 the *in vivo* clearance of biotinylated red blood cells in both WT and Gas6^{-/-} mice was not affected by rGas6 treatment (percent biotinylated red blood cells after 5 days: 53 ± 3% in WT and 55 ± 7% in Gas6^{-/-} mice; at day 8: 39 ± 2% in WT and 37 ± 2% in Gas6^{-/-} mice; n = 6, P = n.s.)

Administration of Epo entirely protected WT mice from PHZ-induced
15 anemia (Figure 4a), and in fact, resulted in an elevated hematocrit from day 6 onward. Based on our previous findings of higher Epo levels in Gas6^{-/-} mice in spite of a lower hematocrit and reticulocyte response, it was not expected that Gas6^{-/-} mice would respond to Epo. While over the entire study period, Epo did not have a significant effect (P = 0.094), Epo did induce a significant increase in
20 the hematocrit of the Gas6^{-/-} mice at day 3 (P < 0.001), and a moderate improvement at days 1 and 6 (P = 0.04, P = 0.06, respectively) as compared with Gas6^{-/-} saline treated controls (Figure 4b). But, hematocrit levels remained significantly less than in Epo-treated WT mice at all time points (P < 0.001). This suboptimal response to Epo occurred in concert with reduced reticulocyte
25 counts (reticulocyte index: 30 ± 4% in WT versus 11 ± 1% in Gas6^{-/-}, n = 6, P = 0.007 at day 3 and 50 ± 3% in WT versus 20 ± 4% in Gas6^{-/-} at day 6, n = 6, P = 0.04).

Administration of rGas6 to Gas6^{-/-} mice also provided significant protection against PHZ hemolysis-induced anemia (P = 0.003) over the entire
30 study period, with significant benefit specifically observed at days 3 and 6 (P < 0.02) when endogenous Epo levels were elevated (Figure 4b). Notably, at the dose given, rGas6 was also remarkably effective at protecting WT mice from

PHZ-induced anemia over the study period ($P = 0.001$), and most significantly at day 3 ($P < 0.001$), largely interfering with the typical early profound decrease in hematocrit (Figure 4a). While Epo alone, as compared with rGas6 alone, resulted in significantly higher hematocrits in the WT mice at days 3, 6, and 9, rGas6 yielded an essentially normal hematocrit by day 6, and did not cause the hematocrit to rise above the normal pretreatment level, as was seen with Epo (Figure 4a). Overall, the data indicate that administration of soluble rGas6 enhances erythropoiesis in WT mice during anemia and, importantly, without causing excess erythrocytosis. All mice tolerated the rGas6 treatment without any observable side-effects.

Next it was evaluated whether Epo and rGas6 have a synergistic effect *in vivo*. In WT mice, no difference was observed between the treatment with Epo alone or Epo + Gas6 (Figure 5a). When Gas6^{-/-} mice were treated with the combination of rGas6 plus Epo, an obvious synergistic effect of the two agents on erythropoiesis was observed. The erythropoietic activity of Epo was completely restored such that there was a robust increase in the hematocrit from day 3 - a significantly better response than with Epo alone ($P < 0.001$) - and reticulocyte counts reached levels observed with similarly treated WT mice (reticulocyte index: $31 \pm 5\%$ in WT versus $35 \pm 5\%$ in Gas6^{-/-}, $n=6$, $P = \text{n.s.}$ at day 3 and $44 \pm 5\%$ in WT versus $55 \pm 5\%$ in Gas6^{-/-} at day 6, $n = 6$, $P = \text{n.s.}$) (Figure 4a, b).

The therapeutic potential of rGas6 was not restricted to the PHZ-induced anemia model. rGas6 also stimulated erythropoiesis in WT mice after bleeding (500 μl day 0 and 1). Indeed, mice receiving rGas6 (2 μg daily) developed significantly less severe anemia than those receiving saline (rGas6 versus saline control: $P < 0.001$ over the 9-day study period), most notably apparent at days 3 and 6 ($P < 0.005$; Figure 4c). Furthermore, and similar to the effect observed with the PHZ-induced anemia model, treatment with Epo or rGas6 were both effective at day 3, although Epo alone increased the hematocrit levels more than rGas6 alone ($P < 0.001$), specifically evident at days 6 and 9 (Figure 4c), and ultimately to a hematocrit significantly above normal ($P < 0.02$). Treatment with the combination of rGas6 and Epo provided a response almost

identical to that of Epo alone ($P = 0.61$).

EXAMPLE VIII: TREATMENT OF CHRONIC ANEMIA WITH GAS6

Different mice models with chronic anemia have been described.

5 Subtotal nephrectomy in mice results in uremic mice with chronic anemia. They have stable elevations in blood urea nitrogen (mean levels rise from normal range of 26 mg/dl up to a mean of 85 mg/dl) and stable decreases in hematocrit from the normal range (approximately 45%) to approximately 33%. The well-established procedure is performed by surgically removing one kidney, and

10 resecting 2/3 of the other (Hamamori *et al.* (1995) J Clin Invest.95, 1808-1813). These mice are known to respond to recombinant or plasma-derived Epo, with persistent increase in hematocrit to levels above normal - up to 68% (Hamamori *et al.* (1995), cited above). The transgenic mouse strain 134.3LC (Epo-TAg^H) displays a severe chronic anemia resembling that observed clinically during

15 chronic renal failure, with a stable chronic anemia due to a relative deficiency in erythropoietin. Mice homozygous for the modified *Epo* allele have hematocrits in the range of 13-20%, but are otherwise healthy appearing. With administration of exogenous murine Epo, they initially respond with a prominent rise in hematocrit, to levels above normal. Subsequently (after 3-5 weeks of

20 treatment), the response diminishes and their hematocrits fall, as they develop an immune response against endogenous and exogenous Epo (Rinsch *et al.* (2002) cited above). A third model of a disease associated with chronic anemia is that of mice with beta-thalassemia (Bohl *et al.* (2000) Blood 95, 2793-2798). These mice have been shown to respond to erythropoietin delivered via gene

25 therapy routes, with hematocrits rising to normal or above normal range, depending on how much Epo is delivered.

Mice have been generated that represent the anemia of chronic renal insufficiency with low level of Epo and a chronic anemia. These mice do respond to exogenous and endogenous Epo. The mice will be provided for

30 study by the lab of Dr. Patrick Maxwell, Imperial College London.

Mice of each of these models with chronic anemia are treated with a range of daily intraperitoneal doses of rGas6 (from 0.2 ug/day to 10 ug/day)

and/or recombinant human Epo (Epo) ranging in doses from 0.5 IU to 10 IU every other day intraperitoneally. Experiments are performed over a period of up to 8 weeks. Hematocrits, reticulocyte counts and serum Epo levels are monitored every 2-3 days from blood obtained by retro-orbital puncture.

5 The same experiments are performed on mice with acute anemia (PHZ-induced hemolytic mice, see example VII).

 The minimum dose of rGas6 or Epo required to achieve a stable, normal (or at least target) hematocrit levels is established. It is confirmed that rGas6 does not result in polycythemia in these models, whereas Epo does induce
10 polycythemia.

EXAMPLE IX: ESTABLISHMENT OF THE OPTIMAL GAS6/EPO COMBINATION

 In the previous experiment the minimum doses of recombinant Gas6 and
15 Epo that are individually necessary to attain a normal hematocrit in the different models of anemia. In a next set of experiments, Gas6 and Epo are administered in combinations to these mice.

 The minimum doses of Gas6 and Epo that provided full restoration or protection from anemia are first administered in combination. The hematocrit is
20 demonstrated in some cases to rise to above normal under these conditions, due to the effects of Epo. In separate experiments, the amounts of rGas6 and Epo are decreased, identifying different combinations that restore normal hematocrit levels or provide protection, while not inducing polycythemia. It is shown that a combination of doses of Epo and rGas, each below that which
25 would be required for a therapeutic response when given individually, cures or is protective from anemia, while not inducing polycythemia.

EXAMPLE X: GAS6 IN THE TREATMENT OF ANEMIA IN MICE RESISTANT TO EPO

30

 The transgenic mouse strain 134.3LC (Epo-TAgH) displays a severe chronic anemia resembling that observed clinically during chronic renal failure, with a

stable chronic anemia due to a relative deficiency in erythropoietin. Mice homozygous for the modified Epo allele have hematocrits in the range of 13-20%, but are otherwise healthy appearing. With administration of exogenous murine Epo, they initially respond with a prominent rise in hematocrit, to levels
5 above normal. Subsequently (after 3-5 weeks of treatment), the response diminishes and their hematocrits fall, as they develop an immune response against endogenous and exogenous Epo (Rinsch *et al.* (2002) *Kidney Int.* **62**, 1395-1401).

Transgenic Epo-TAgH mice are treated with daily intraperitoneal doses of
10 rGas6 (from 0.2 ug/day to 10 ug/day) or recombinant human Epo (Epo) ranging in doses from 0.5 IU to 10 IU every other day intraperitoneally.

In the Epo-treated Epo-TAgH mice, an initial rise in hematocrit is observed, with levels above normal, followed by a gradual decrease in hematocrit.

15 In the Gas6 treated mice, a gradual rise in hematocrit is observed up to a normal level, which remains stable up to 12 weeks after initiation of the therapy.

EXAMPLE XI: EFFECT OF EPO ALONE OR IN COMBINATION WITH RECOMBINANT GAS6 ON CHRONIC ANEMIA

20 Patients suffering from chronic renal failure develop anemia due to inadequate erythropoietin (Epo) production by the kidney. Recombinant Epo, in some patients, partially restores hematocrit and blood hemoglobin concentration, decreasing the need for blood transfusions. Epo alone is not always effective.

25 Animal models using partial nephrectomy have been developed to reproduce conditions that lead to anemia in patients with chronic renal failure. Nevertheless, these models suffer from shortcomings, including hematocrits that do not decrease to the levels observed in chronic renal failure and toxicity due to renal failure conducting animals to death (Hamamori, Y. *et al.* 1995, *J Clin Invest*, 95:1149-1162).
30

A transgenic mouse strain (134.3 LC, *Epo-Tag^H*), which has a stable chronic anemia due to deficiency of erythropoietin, was used [Maxwell *et al.*

(1993) *Kidney Int* **44**, 1149-1162]. This mouse model constitutes a valuable tool for refining gene transfer approaches for delivery of Epo. Mice homozygous for the modified Epo allele are severely anemic, with hematocrits ranging from 13% to 25%, but otherwise appear healthy and remain active. Heterozygous mice are moderately anemic with hematocrit levels below 45% but higher than 25%. Homozygous mice display hematocrit levels comparable to those observed in patients with chronic renal failure.

Mice that are heterozygous for *Epo-Tag^H*, were treated with either Epo alone (10 IU/day) or Gas6 alone or with Epo 10 IU/day plus recombinant Gas6 2 µg per day (see Figure 5). When mice were injected with 2 µg of rGas6 alone per day, a dose that was shown to be effective in restoring hematocrit in acute anemia models, we did not observe an effect after 8 days of treatment. We therefore increased the dose of rGas6 to 4 microgram per day and then obtained a significant increase in hematocrit already after 4 days. Co-administration of rGas6 2 µg per day with Epo 5 IU/day resulted in a slightly higher hematocrit over that of either drug administered alone. The synergistic beneficial effect became significant when low dose rGas6 (2 microgram per day) was administered together with Epo (10 IU per day), resulting in a rise in the hematocrit higher than Epo alone or rGas6 alone. Thus, Gas6 is effective in chronic anemia, augments the effects of Epo, and may have Epo dose-sparing effects.

These results indicate that the addition of Gas6 to Epo, for treatment of anemia, has a greater beneficial effect over Epo alone. These results also indicate that combination therapy with Gas6 + Epo will be therapeutically beneficial in the treatment of patients with anemia.

EXAMPLE XII: GAS6 IN THE TREATMENT OF ANEMIA IN PATIENTS RESISTANT TO EPO

A subgroup of anemic patients suffering from chronic renal failure, which do not respond to Epo are treated with an effective dose of Gas6 for 4 weeks. At the end of this time period it is established that the hemoglobin level of these patients has normalized.

CLAIMS

1. The use of a Gas6 compound for the manufacture of a medicament for the treatment or prevention of anemia.
- 5 2. The use according to claim 1 wherein the Gas6 compound is Gas6 protein or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative.
- 10 3. The use of biologically active substances inducing a Gas6 expression or release for the manufacture of a medicament for the prevention or treatment of anemia.
- 15 4. The use according to claim 3, wherein the biologically active substance is a vector comprising the Gas6 gene.
5. The use according to any of the claims 1 to 4, wherein said treatment or prevention is the treatment or prevention of a patient susceptible to the adverse side-effects of Epo.
- 20 6. The use according to claim 5, wherein said patient susceptible to the adverse side-effects of Epo is a patient suffering from hypertension.
7. The use of a Gas6 compound for the manufacture of a medicament for the treatment or prevention of anemia in a patient irresponsive to Epo.
- 25 8. The use according to claim 7 wherein the Gas6 compound is Gas6 or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative.
- 30 9. The use of a Gas6 compound in combination with erythropoietin or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated

salt of said EPO derivative for the manufacture of an antianemic drug or an antianemic composition.

10. The use according to claim 9, wherein the Gas6 compound is Gas6 or an
5 analogue, mutant or derivative thereof, or a physiological tolerated salt of
said Gas6 derivative,

11. The use according to claim 10, wherein said antianemic drug is a drug for
the treatment or prevention of anemia in a patient for which treatment with
10 Epo is contra-indicated.

12. The use of claim 10, wherein said antianemic drug is a drug for the
treatment or prevention of anemia in a patient irresponsive to Epo or an
analogue, mutant, variant or derivative thereof, or a physiologically tolerated
15 salt of said EPO derivative.

13. A method for the treatment, curing or prevention of anemia in a patient
which is irresponsive to Epo, which method comprises: administering thereto
an optimized combination of a Gas6 compound and erythropoietin or an
20 analogue, mutant, variant or derivative thereof, or a physiologically tolerated
salt of said EPO derivative, either simultaneously or sequentially, said
optimized concentration ensuring a target or normalized hemoglobin level.

14. The method according to claim 13 wherein the Gas6 compound is Gas6 or
25 an analogue, mutant or derivative thereof, or a physiologically tolerated salt
of said Gas6 derivative.

15. A method for the treatment, curing or prevention of anemia in a patient for
which treatment with Epo is contra-indicated, which method comprises:
30 administering to said patient an optimized combination of a Gas6 compound,
and erythropoietin or an analogue, mutant, variant or derivative thereof, or a
physiologically tolerated salt of said EPO derivative, either simultaneously or

sequentially, said optimized concentration ensuring a target or normalized hemoglobin level.

16. The method of claim 15, wherein the Gas6 compound is Gas6 or an
5 analogue, mutant or derivative thereof, or a physiologically tolerated salt of said Gas6 derivative.
17. An antianemic pharmaceutical composition comprising a Gas6 compound,
or an optimized formulation of such a Gas6 compound with erythropoietin or
10 an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative.
18. The composition according to claim 17, wherein the Gas6 compound is Gas
or an analogue, mutant or derivative thereof, or a physiological tolerated salt
15 of said Gas6 derivative.
19. A method of selection of a formulation of a pharmaceutical ratio of a gas6
compound to Epo or an analogue, mutant, variant or derivative thereof, or a
physiologically tolerated salt of said EPO derivative, said method
20 comprising:
- a) determining the minimal dose of Epo or an analogue, mutant, variant or derivative thereof, or a physiologically tolerated salt of said EPO derivative which does not induce adverse side-effects in a patient; and
 - b) administering in addition to a) incremental doses of a Gas6 compound so
25 as to determine which combined Epo/Gas6 dosage ensures maintenance of target hemoglobin levels in said patient
 - c) selecting the ratio in accordance with the determination of step b).
20. The method according to claim 19, further comprising preparing a
30 pharmaceutical composition comprising the selected ratio of the EPO or the analogue, mutant, variant or derivative thereof, or the physiologically tolerated salt of said EPO derivative to the Gas6 compound.

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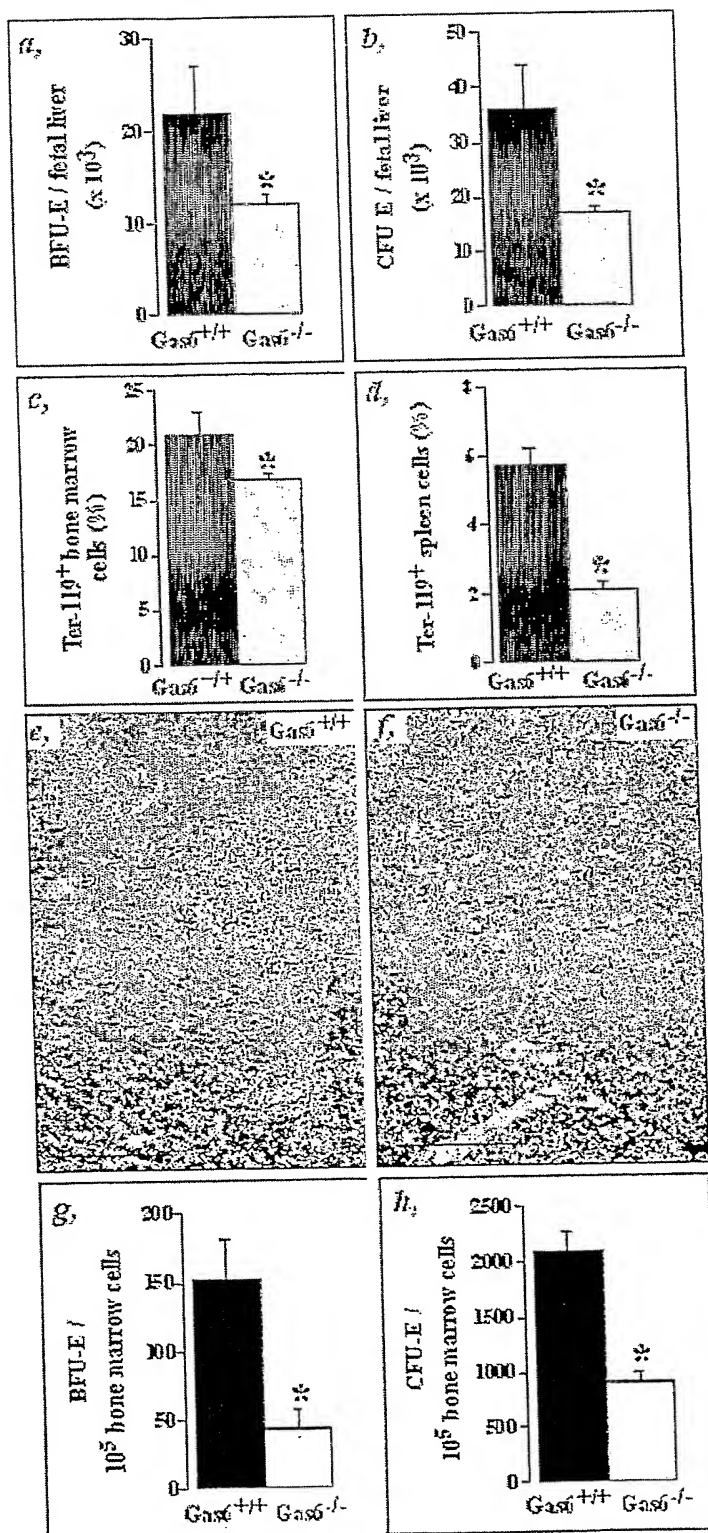


Fig. 1

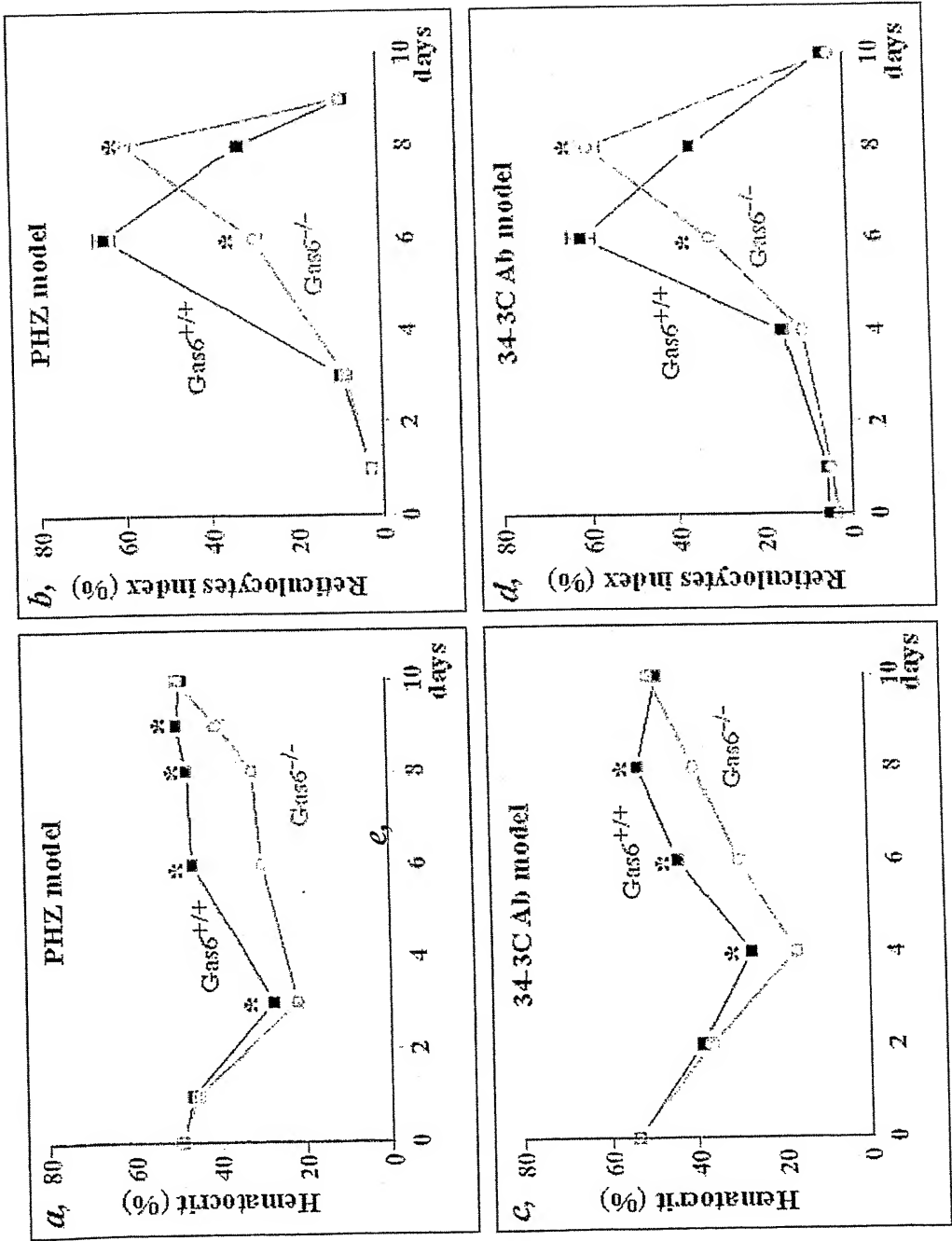


Fig. 2

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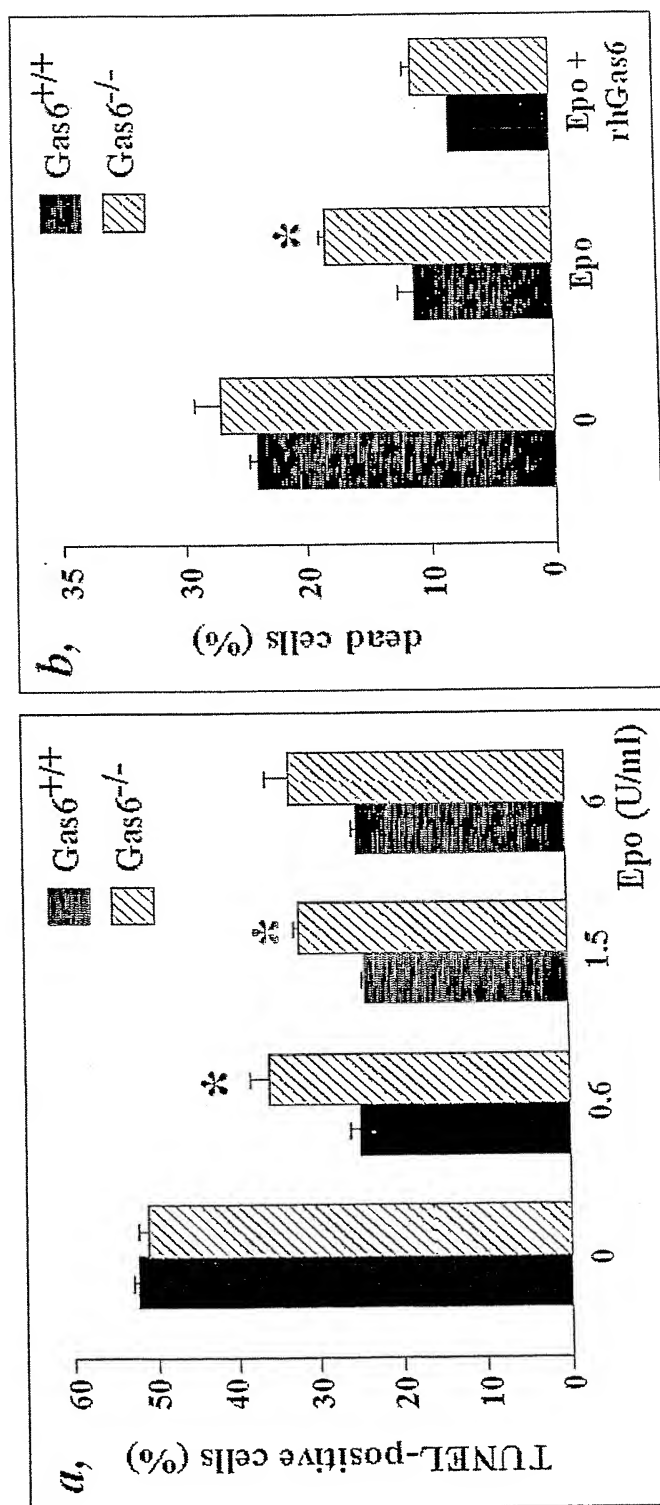


Fig. 3

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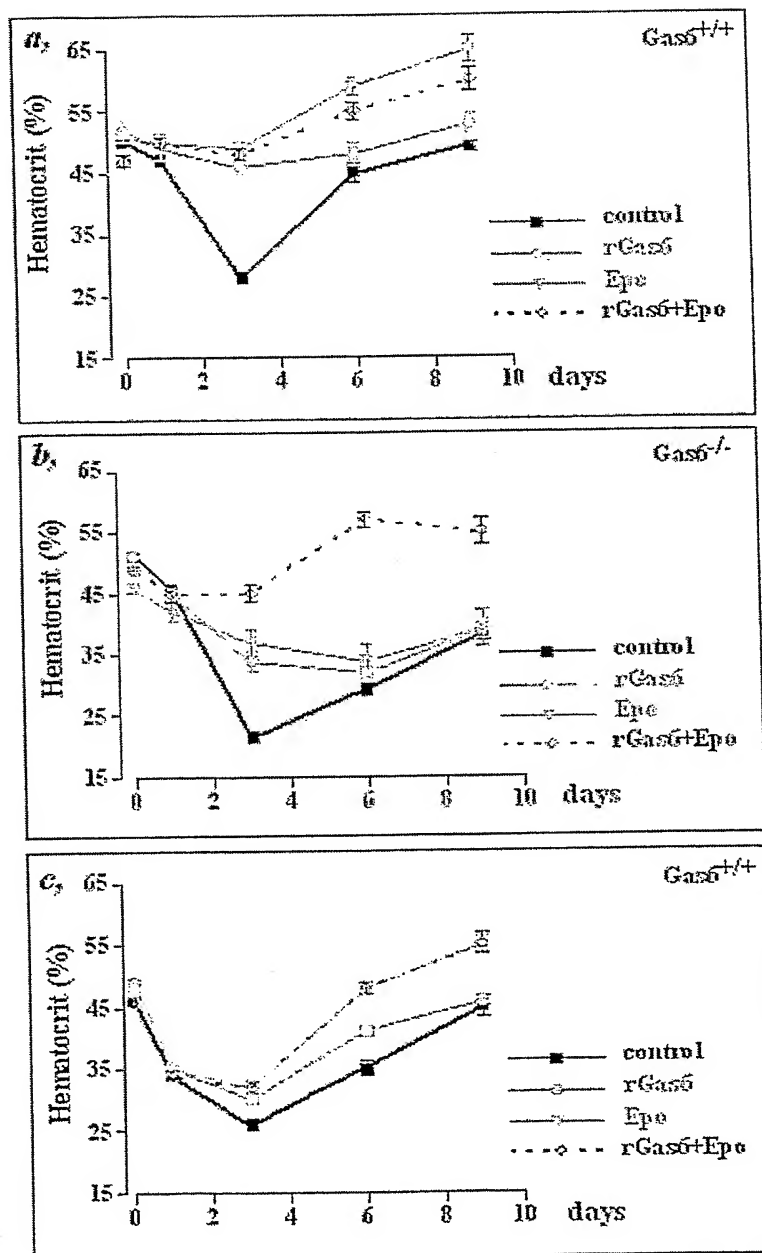
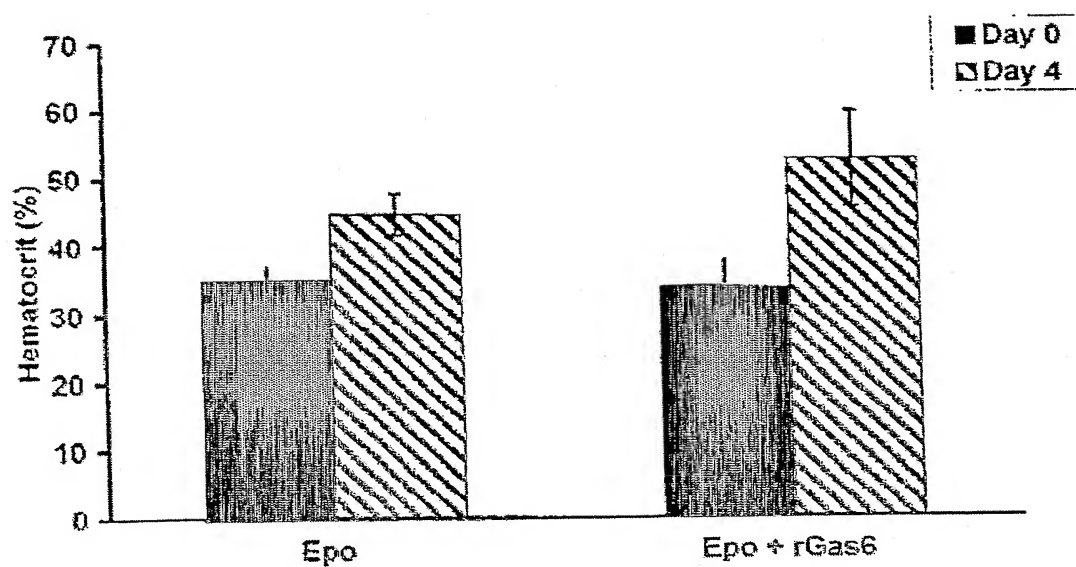


Fig. 4

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Epo: 10 IU/day

rGas6: 2 microgram/day

Figure 5

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[Continued on next page]

(54) Title: TREATMENT OF ANEMIA



Epo: 10 IU/day

rGas6: 2 microgram/day

(57) Abstract: The present invention relates to a method for the treatment of anemia based on the administration of the product of growth arrest-specific gene 6 (Gas6), its mutants, variants, active derivatives and the physiological tolerated salts of said Gas6 derivatives or of a group of biologically active substances inducing a GAS6 expression or releasing action or of a group of compounds that activate the Sky, Axl or Mer receptor tyrosine kinases. Furthermore the invention relates to a pharmaceutically effective composition to treat anemia. The composition comprises a pharmaceutically effective amount of a product of growth arrest-specific gene 6 (Gas6), or its mutant, variant, active derivative or the physiological tolerated salts of said Gas6 derivative or a biologically active substance that induces GAS6 expression or GAS6 release or a substance that activates the Sky, Axl or Mer receptor tyrosine kinases.



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EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, PASCAL

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 378 899 A (THROMB X N V) 26 February 2003 (2003-02-26) the whole document	1-20
X	WO 96/28548 A (CHEN JIAN ; GENENTECH INC (US); GODOWSKI PAUL J (US); LI RONGHAO (US);) 19 September 1996 (1996-09-19) page 34, line 7 - line 8 page 35, line 21 ----- -/--	1-20

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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